STUDIES ON THE UREASE OF THE EGGS AND EMBRYOS OF THE SEA URCHIN, STRONGYLOCENTROTUS PURPURATUS [1]

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From studies on the comparative physiology of animals, it has been found that the kind of nitrogen excretion products tend to be correlated with environmental conditions, the most important single factor being the availability of water. One might suppose, on the basis of environment, that if sea urchin eggs excrete nitrogen it would be excreted in the form of ammonia since ample water is available for the rapid removal of this substance from the eggs and developing embryos. There are, in fact, reports of ammonia excretion by sea urchin eggs. Örström (1941) demonstrated a marked but transitory excretion of ammonia by sea urchin eggs immediately after fertilization and provided some evidence that this is a result of the deamination of adenosine to inosine by adenosine deaminase. Hutchens *et al.* (1942) confirmed Örström's findings, but attributed the ammonia production to amino acid deamination. A number of workers have investigated nitrogen metabolism during later development with results which, though in some measure conflicting, suggest that the early development of the sea urchin egg is characterized by little if any nitrogen excretion. Hayes (1934) reported an increase in total nitrogen during the first four hours of the development of *Echinometra lucunter*, followed by a small decrease between the 6th and 24th hours of development. Ephrussi and Rapkine (1928) found a decrease in total nitrogen from 10.7% dry weight to 9.7% during the period of development between 0–40 hours of *Paracentrotus lividus*. Hayes' data are complicated by the possibility that variable amounts of the gelatinous coat of the eggs, a glycoprotein (Tyler, 1949a), may have been present in different stages, since no special account of this seems to have been taken in this work. Ephrussi and Rapkine's results, as indicated by Horowitz (1939), also show an increase of 5.6% in the dry weight of the embryos during the period of development mentioned which would virtually cancel out any nitrogen loss during this period. Horowitz (1939) presented data for the nitrogen content of the eggs of *Urechis caupo*, a marine echiuroid worm, indicating that the nitrogen content is constant up to 24 hours of development (feeding trochophore). Though this last mentioned work admittedly has no direct bearing on nitrogen excretion in the sea urchin egg, the environment of the two forms is similar and one might expect, therefore, a similarity in nitrogen excretion products. Finally, it is reported by Gustafson and Hasselberg (1951) that the nitrogen content of developing embryos of *Paracentrotus lividus* remains constant for the first 32 hours of development.

[1] This investigation was supported in part by the Research Fund 171 of the University of Washington.

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The present investigation is concerned with the enzyme urease and metabolically related enzymes, and the possible relation between these enzymes and the low nitrogen excretion by the developing sea urchin embryo. Since urease is usually thought of as an "excretory" enzyme, its existence in an embryo which may not excrete nitrogen during the morphogenetic period leading to the larval stage becomes of some interest.

Materials and Methods

Eggs and sperm of *Strongylocentrotus purpuratus* were collected after the animals had been induced to spawn with isotonic KCl (Tyler, 1949b). The gelatinous coat of the eggs was removed with acid (pH 3.5) sea water (Tyler, 1949a), and the eggs were used if they were 90–100% fertilizable. Embryos were grown in approximately 1% suspensions at 11° C. in slowly rotating Erlenmeyer flasks. At the desired stage of development, samples were taken from the suspension. Swimming stages were concentrated by means of a specially constructed Lucite centrifuge similar to the Foerst plankton centrifuge. The embryos were frozen and stored for not more than 24 hours before *in vitro* assay for urease activity.

For *in vitro* determinations, the frozen material was homogenized and diluted with sea water to give a final concentration of about 0.5 ml. of packed eggs per 2.0 ml. of brei. Two milliliters of this were transferred to a 10-ml. screw-cap vial with 4.0 ml. of 0.5 *M* phosphate buffer at pH 7.4. At zero time, 1.0 ml. of a urea solution containing 100 mg. of urea was added. The reaction was allowed to proceed for one hour at 29.5° C., when the vials were chilled and centrifuged for 2–3 minutes at 3° C. Five milliliters of the supernatant were transferred to tubes containing 1 gm. of Permutit (after Folin). These were mixed for one minute, then washed three times with ammonia-free water. The Permutit was transferred to a Kirk-type micro-Kjehldahl still and alkalinized by the addition of 3 ml. of 20% NaOH. The ammonia liberated was distilled and titrated by the biiodate method of Ballentine and Gregg (1947). Kjehldahl nitrogen was determined on aliquots of the homogenates by the digestion method of Boell (1945) and the distillation and titration method of Ballentine and Gregg (1947).

For *in vivo* determinations, 6-ml. aliquots containing about 0.5 ml. of eggs were placed in a 10-ml. screw-cap vial, one ml. of sea water containing 100 mg. of urea was added and the eggs incubated for one hour at 18° C. The suspensions were then centrifuged lightly at 3° C. for one minute and 5.0-ml. aliquots of the supernatant were transferred to the Permutit. The eggs were washed with sea water, allowed to cytolyze in the original volume of cold distilled water and another series of 5.0-ml. aliquots taken from the supernatant. The cytolysis procedure permitted measuring ammonia that was produced by the reaction but failed to diffuse out of the eggs. The *in vivo* urease activity is taken as the sum of the ammonia liberated to the sea water plus that bound in the eggs and is expressed as micrograms of ammonia nitrogen per aliquot per hour.

Endogenous controls were run in all experiments and the data to be presented have been corrected by the subtraction of the control values. The endogenous ammonia production in breis was generally about 0.0075 microgram ammonia nitrogen per microgram egg nitrogen per hour, while for whole eggs, the usual figure was close to 17 micrograms ammonia nitrogen per aliquot per hour for unfertilized eggs

and 20 for fertilized, varying slightly with the number of eggs per aliquot present in different experiments.

Results

To ascertain the *in vitro* saturation point of the enzyme with substrate, two experiments with equal aliquots of eggs and varying urea concentrations were performed. The amount of urea per vial ranged from 2.5 to 20 mg. in one experiment, and from 10 to 90 mg. in the second experiment. The results indicate complete saturation with 30–50 mg. of urea but, as a safety factor, 100 mg. of urea per reaction vial were used in all *in vitro* determinations of urease activity.

Since urease in other organisms depends on reduced sulfhydryl groups for activity (Sumner and Poland, 1933), a check was made on the redox potential of the brei-buffer mixture using a Beckman pH meter equipped with a platinum electrode. The brei-buffer was found to have a redox potential of 150–170 mv. On the addition of one mg. of glutathione, this was lowered to 90–100 mv. The enzymatic activity was not appreciably changed in the presence of glutathione; values obtained were 0.0365 microgram ammonia nitrogen per microgram egg nitrogen per hour without glutathione and 0.0380 for the vial containing glutathione. As will be seen later, these results do not differ significantly from each other. In view of the failure of glutathione to increase the activity of the enzyme, and in view of the optimum redox potential of 100–200 mv. reported by Sizer and Tytell (1941) for jack bean urease, which is within the range obtained for the brei-buffer mixture, glutathione was not included in the subsequent assays.

To determine the reproducibility of the homogenizing, sampling and assay methods employed, embryos were raised to the hatching blastula stage and harvested. Three aliquots of these embryos were homogenized separately and their urease activities determined. Activities of 0.0361, 0.0368, and 0.0328 unit (microgram ammonia nitrogen/microgram egg nitrogen hour) were obtained, from which it is concluded that the determinations are reliable to about 0.005 unit.

Using the conditions for assay established by the above experiments, determinations of the activity of the enzyme were made on breis of fertilized and unfertilized eggs and on various stages during the first 114 hours of development. These results are summarized in Figure 1. It is seen that while there is little change in the activity of breis between fertilized and unfertilized eggs, there is a drop of about 30% by the unhatched blastula stage, and that this level is maintained to the pluteus stage, at which time the animals are beginning to feed. Adult tissues were analyzed in order to see if any further change in the activity of the enzyme occurred. Ovary, from which the eggs had been removed by KCl-induced spawning, and gut were assayed. Activities of 0.0285 and 0.0585 unit, respectively, were found. It would seem that the activity of urease in the adult animal is maintained at approximately the level found during embryonic development.

That urease activity *in vitro* does not change when the eggs are fertilized is indicated above. There still exists the possibility of an *in vivo* change. To test this, equal aliquots of eggs were assayed in the manner described above. For two separate experiments, the results are: experiment 1, 126.8 micrograms ammonia nitrogen per hour per aliquot unfertilized and 99.9 fertilized; experiment 2, 127 unfertilized and 119 fertilized. These experiments show little or no change in *in vivo* urease

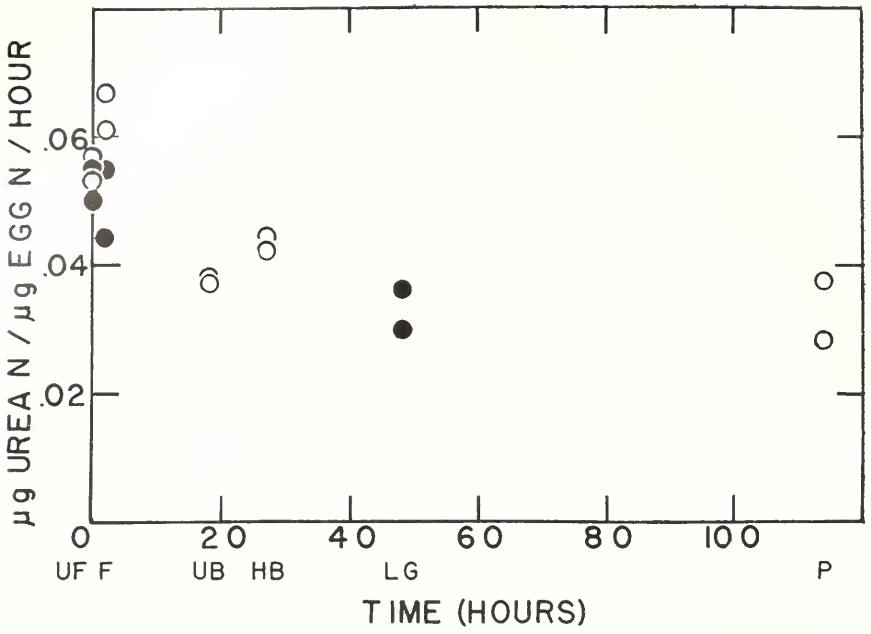

FIGURE 1. The *in vitro* activity of urease of *Strongylocentrotus purpuratus* as a function of time and developmental stage. UF, unfertilized; F, fertilized; HB, hatched blastula; LG, late gastrula; P, pluteus.

activity accompanying fertilization; the differences noted are certainly not comparable to the 4- to 6-fold increase found in respiratory rate on fertilization in sea urchins.

Experiments were done to determine if a change in urease activity resulted from homogenization. Equal aliquots of a suspension of unfertilized eggs were taken; one series was homogenized and the other was left intact. The temperature was 18° C. and the pH was 6.8 for the homogenized samples and 7.8 for the sea water in which the intact eggs were suspended. Though there is an apparent difference in the pH of the two samples, the enzyme may be considered to be acting at the same pH in both cases since the pH of the interior of the egg may be taken to be about 6.8 (Pandit and Chambers, 1932). The intact eggs produced 70 micrograms of ammonia nitrogen per hour per aliquot while the homogenized eggs produced 238.

There are numerous possible explanations for this four-fold increase in activity. One of these, the possibility that the eggs are relatively impermeable to urea, was tested by assaying for unfertilized *in vivo* activity with increasing amounts of urea. If urea is freely permeable, the *in vitro* saturation point (30 mg.) should also be reached *in vivo*. Two such experiments were done employing urea concentrations from 10 to 100 mg. per vial. The results, plotted in Figure 2, indicate that the enzyme is not saturated at 100 mg. of urea per vial (0.24 *M*) and therefore that the amount of substrate that reaches the enzyme is limited, presumably by permeability, assuming that urea is not being utilized by the eggs during the experiment. It should be noted that eggs incubated with urea were fertilizable to the same extent as endogenous controls (75–80%) after one or two washings with fresh sea water.

In order to have a more complete picture of the role of urease, it would be helpful to know how urea might be formed in the eggs. There are several pathways of urea formation, of which two are the ornithine cycle and the degradation of purines. Since the presence of a system such as the ornithine cycle, which would manufacture urea at the expense of energy only to have it immediately broken down again, seemed unlikely, it was decided to test for various enzymes of purine degradation. Homogenates of unfertilized eggs were examined qualitatively for the presence of adenase, xanthine oxidase, uricase, allantoinase, and allantoicase. Adenase was not demonstrable in more than trace amounts in two independent experiments using the method employed for urease, but with adenine as substrate. Xanthine oxidase was tested for using a modified Thunberg technique with both xanthine and hypoxanthine as substrates. Both substrates gave decolorization times of 10–15 minutes at 29° C. and pH 7.4, while in the absence of substrates, decolorization did not occur within 40 minutes. It is concluded that the enzyme is present. Uricase activity was measured in a Warburg apparatus. Two ml. of brei (0.5 ml. packed eggs per 2 ml.), to

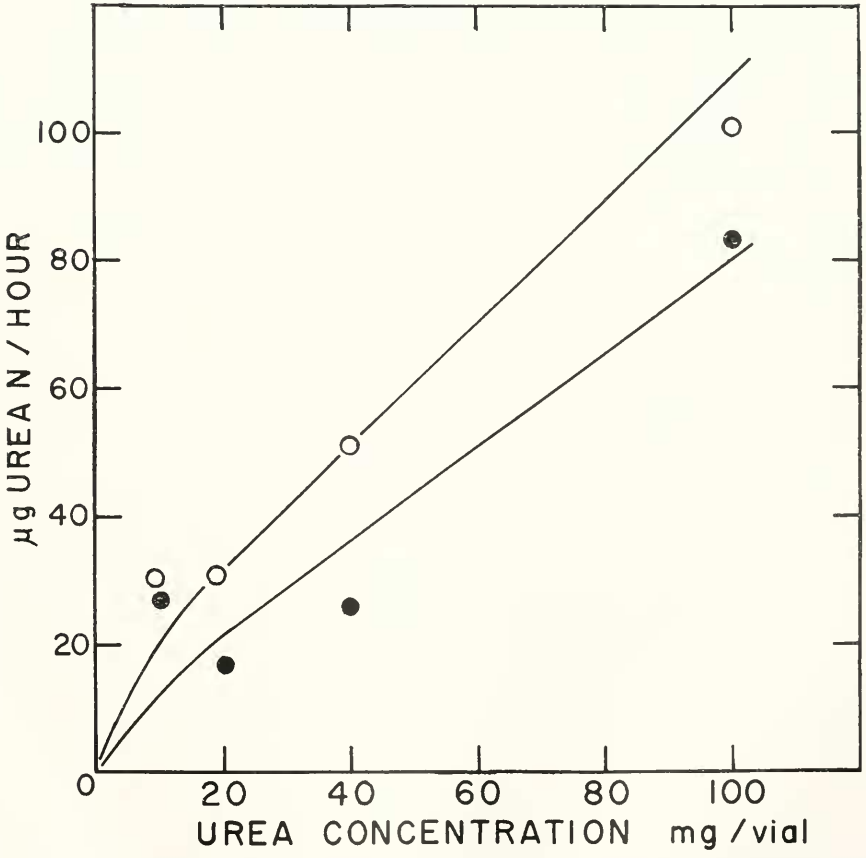

FIGURE 2. The *in vivo* activity of urease in unfertilized eggs of *Strongylocentrotus purpuratus* as a function of concentration of urea in the sea water.

which were added 20 mg. uric acid, gave an oxygen uptake at pH 9 and 30° C. of 10.0 and 14.0 microliters of O_2 per hour per flask in two separate experiments, whereas endogenous controls showed rates of 3.1 microliters of O_2 per hour per flask in both experiments; uricase is therefore present in the brei. Allantoinase and allantoicase were assayed simultaneously using the *in vitro* urease method, but with allantoin as substrate. Duplicate reaction vials contained 5.0 ml. McIlvaine's buffer at pH 7.4, 1.0 ml. undiluted egg homogenate, and 1.0 ml. allantoin solution containing 5 mg. of allantoin. In the duplicates 97.2 and 87.1 micrograms ammonia nitrogen were liberated in one hour at 22° C. These values have been corrected for a small endogenous ammonia production. It is concluded that allantoinase and allantoicase are present.

These experiments indicate that four of the enzymes metabolically related to urease are present in the unfertilized eggs. In addition, adenosine deaminase has been reported by Örström (1941) and Gustafson and Hasselberg (1951). It is probable, therefore, that the scheme of purine degradation typical of animal tissues exists in these eggs. With this conclusion, the function of urease in the sea urchin embryo might be expected to be that of completing the degradation of purines to ammonia, preparatory to its elimination from the organism as in a non-cleidoic system where water supply is not limiting. Against this, however, are the investigations cited earlier that indicate little or no nitrogen is lost by sea urchin embryos. The question of ammonia excretion in *Strongylocentrotus purpuratus* was examined directly by experiments in which the ammonia liberated by embryos into the sea water culturing medium was measured. One experiment covered the period from 20 minutes to 12 hours after fertilization, and a second was concerned with the interval from 24 to 60 hours after fertilization. In both, a 0.2% suspension of eggs was inseminated and, after washing, was cultured for the incubation period in fresh sea water at 11° C. and pH 8. Ammonia was determined on 5.0-ml. aliquots of the sea water both before and after the incubation period. No detectable ammonia was liberated by the larvae during the developmental periods tested.

With the establishment of the likelihood that nitrogen is excreted in very small quantities, if at all, during the early development of the sea urchin, it follows that urease is not primarily involved here with ammonotelic excretion. It is probable, then, that any ammonia produced by urease would be added to the metabolic pool of nitrogen to be used in the synthesis of various nitrogen compounds. Hultin (1950) has shown that the eggs of *Paracentrotus lividus* are able to incorporate N^{15}-labelled ammonia from sea water into proteins, a finding which tends to indicate the existence of such a metabolic pool. In this view, urease and the other enzymes of purine breakdown shown to be present are to be considered as factors in the mobilization of nitrogen for the many syntheses of the sea urchin embryos. An interesting possible source of purine for their action may be ribonucleic acid, with which these eggs are richly supplied.

Summary

1. The urease activity of sea urchin eggs and embryos has been followed *in vitro* to the pluteus stage. The activity was found to decline by about 30% upon reaching the blastula stage, after which it remains relatively constant.

2. Adult sea urchin tissues were found to contain urease of activity comparable to that of the eggs and embryos.

3. The *in vivo* activity of urease in fertilized and unfertilized eggs was compared, with little or no change on fertilization noted.

4. The *in vivo* activity was found to be considerably lower than *in vitro* activity when measured under the same conditions, and evidence indicates that the *in vivo* activity is limited, at least in part, by the rate of penetration of urea into the eggs.

5. Of the enzymes usually involved in the formation of urea from purines, xanthine oxidase, uricase, allantoinase and allantoicase are shown to be present in sea urchin eggs, whereas adenase was not demonstrable with the method employed.

6. No ammonia within the range detectable by the methods employed was liberated into sea water by embryos during the periods of 20 minutes to 12 hours after fertilization, and 24 hours to 60 hours after fertilization.

7. The results suggest that urease and the related enzymes function to add nitrogen from purines to the metabolic ammonia pool during the pre-feeding stages of development.

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